Local Vitamin D Metabolism in Bone and Muscle

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2017

CHAPTER 5

Effects of $1,25(OH)_2D_3$ and $25(OH)D_3$ on C2C12 Myoblast Proliferation, Differentiation, and Myotube Hypertrophy

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Published in: Journal of Cellular Physiology 2016 Nov; 231(11): 2517-28

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ABSTRACT

An adequate vitamin D status is essential to optimize muscle strength. However, whether vitamin D directly reduces muscle fiber atrophy or stimulates muscle fiber hypertrophy remains subject of debate. A mechanism that may affect the role of vitamin D in the regulation of muscle fiber size is the local conversion of 25(OH)D to 1,25$(OH)_2$D by 1α-hydroxylase. Therefore, we investigated in a murine C2C12 myoblast culture whether both 1,25$(OH)_2D_3$ and 25(OH)D_3 affect myoblast proliferation, differentiation, and myotube size and whether these cells are able to metabolize 25(OH)D_3 and 1,25$(OH)_2D_3$. We show that myoblasts not only responded to 1,25$(OH)_2D_3$, but also to the precursor 25(OH)D_3 by increasing their VDR mRNA expression and reducing their proliferation. In differentiating myoblasts and myotubes 1,25$(OH)_2D_3$ as well as 25(OH)D_3 stimulated VDR mRNA expression and in myotubes 1,25$(OH)_2D_3$ also stimulated MHC mRNA expression. However, this occurred without notable effects on myotube size. Moreover, no effects on the Akt/mTOR signaling pathway as well as MyoD and myogenin mRNA levels were observed. Interestingly, both myoblasts and myotubes expressed CYP27B1 and CYP24 mRNA which are required for vitamin D_3 metabolism. Although 1α-hydroxylase activity could not be shown in myotubes, after treatment with 1,25$(OH)_2D_3$ or 25(OH)D_3 myotubes showed strongly elevated CYP24 mRNA levels compared to untreated cells. Moreover, myotubes were able to convert 25(OH)D_3 to 24R,25$(OH)_2D_3$ which may play a role in myoblast proliferation and differentiation. These data suggest that skeletal muscle is not only a direct target for vitamin D_3 metabolites, but is also able to metabolize 25(OH)D_3 and 1,25$(OH)_2D_3$.

INTRODUCTION

Aging is associated with a loss of muscle mass, bone mass, and strength, which may result in reduced mobility and an increased risk for falls and fractures [14;37]. An adequate vitamin D status is essential to reduce the risk for falls and fractures and to optimize bone mineral density and muscle strength [8;32;33]. Vitamin D stimulates calcium absorption from the intestine and maintains serum calcium levels which is required for normal bone mineralization and muscle function [30]. Regarding bone metabolism, vitamin D reduces osteoblast proliferation, stimulates osteoblast differentiation, and induces RANKL expression in osteoblasts which is involved in stimulation of osteoclast formation and bone resorption [2;30;57]. However, whether vitamin D directly reduces muscle fiber atrophy or stimulates muscle fiber hypertrophy remains subject of debate.

Several in vivo studies suggest a role for vitamin D in the regulation of muscle mass and function. Observational studies demonstrate that vitamin D deficiency in elderly people is associated with reduced muscle mass [54] and strength [7;64], lower physical performance [54;61], and an increased risk of falling [45]. Furthermore, a meta-analysis of 17 randomized controlled trials showed that vitamin D supplementation in subjects with a baseline serum 25-hydroxyvitamin D (25(OH)D) lower than 25 nmol/l did have a positive effect on hip muscle strength [50]. In animal models, reduced muscle function was reported in vitamin D deficient rats [36;38] and chickens [8] compared to control animals. The studies described above suggest that vitamin D can affect muscle mass and function, however it is not clear whether vitamin D plays a direct or indirect role.

In vitro studies on myoblasts and myotubes show that the active metabolite 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) is able to directly affect myogenesis [11;22;24]. Myogenesis, a process that is essential for muscle regeneration, growth and hypertrophy, includes satellite cell activation, myoblast proliferation, differentiation, and myotube formation [65]. Regarding myoblast proliferation in vitro, most studies show inhibitory effects of 1,25$(OH)_2$D [22;24;35;44;46] likely due to a cell cycle arrest at the G1 to S transition [24]. However, 1,25$(OH)_2$D effects on proliferation have also been reported to be absent [48] or stimulatory [11;13;17]. Furthermore, whether 1,25$(OH)_2$D affects myoblast differentiation and hypertrophy of differentiated myotubes is not well known. Recently, it has been shown that when myoblasts were cultured in growth medium and subsequently in differentiation medium which were supplemented with 1,25$(OH)_2$D from the start of the culture resulted in less myotubes [24], but myotubes were larger in diameter than those that were differentiated in medium without supplemented 1,25$(OH)_2$D [22;24]. Since in these experiments the number of myoblasts was not standardized due to the anti-proliferating effects of 1,25$(OH)_2$D, the larger myotube size could not be ascribed to a direct effect of 1,25$(OH)_2$D per se. As, yet it is still unknown what the effects are of 1,25$(OH)_2$D on myotube formation and size in cultures starting with the same number of cells.

A mechanism that may affect the role of vitamin D in the regulation of muscle fiber size and contractile function in vivo is the local conversion of 25(OH)D to 1,25$(OH)_2$D. The metabolite 1,25$(OH)_2$D is primarily synthesized in the kidney from the precursor 25(OH)D [30]. In addition, 1,25$(OH)_2$D synthesis has been demonstrated in several other cell types, such as in osteoblasts [3;28;57;58], prostate cells [40], and monocytes [4]. In osteoblasts, the function of locally synthesized 1,25$(OH)_2$D is supposed to be regulation of cell proliferation and differentiation [3;6;58]. Recent studies have shown that rat muscle as well as C2C12 myoblasts and myotubes also express CYP27B1, which encodes the enzyme 1α-hydroxylase [24;53;46]. Moreover, CYP27B1 activity has indirectly been demonstrated in muscle cells by performing luciferase reporter studies [24] and CYP27B1 silencing experiments [46]. However, to the best of our knowledge, whether C2C12 cells do convert 25(OH)D to 1,25$(OH)_2$D has not been investigated yet. In addition to its possible conversion into 1,25$(OH)_2$D, 25(OH)D may also be converted to 24R,25-dihydroxyvitamin D (24R,25$(OH)_2$D). This conversion is catalyzed by the 24-hydroxylase enzyme, encoded by the CYP24 gene [47]. In osteoblasts in vitro, 24R,25$(OH)_2$D synthesis has been shown [28;56;57] and this metabolite may stimulate cell differentiation through binding to the VDR [15;58]. In myoblasts and myotubes, CYP24 expression has also been shown [24] and CYP24 activity may, therefore, affect skeletal muscle tissue as well. However, whether skeletal muscle cells are capable of synthesizing 24R,25$(OH)_2$D is still unknown.

The aim of this study was to investigate in a murine C2C12 myoblast culture model whether both 1,25$(OH)_2D_3$ and 25$(OH)D_3$ affect myoblast proliferation, differentiation, and myotube size, and which regulatory mechanisms, including myogenic regulatory factors and signaling pathways, are involved. We hypothesized that 1,25$(OH)_2D_3$ affects the expression of genes in the regulation of vitamin D_3 signaling in myoblasts and myotubes, inhibits myoblast proliferation and stimulates myoblast differentiation and myotube hypertrophy. Moreover, we hypothesized that actions of 25$(OH)D_3$ occur via its conversion to 1,25$(OH)_2D_3$. Therefore, we investigated in C2C12 myoblast and myotube cultures whether supplementation of 1,25$(OH)_2D_3$ or 25$(OH)D_3$ to culture medium alters mRNA levels of genes involved in vitamin D metabolism and/or signaling pathways for protein synthesis. We further tested whether myotubes were able to synthesize 1,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ from supplemented 25$(OH)D_3$.

MATERIALS AND METHODS

Cell culture

Mouse C2C12 myoblast cell line was obtained from ATCC (American Type Culture Collection; Manassas, VA, USA). Myoblasts were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM-31885, low glucose, phenol red; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 10 μg/ml penicillin (Sigma–Aldrich, St. Louis, MO, USA), 10 μg/ml streptomycin (Sigma–Aldrich), 50 μg/ml fungizone (Gibco) and incubated at 37°C in humidified air with 5% CO_2. Passages between 4 and 10 were used for experiments and all culture media, including those of treated and control groups, contained 0.1% ethanol (vehicle).

C2C12 myoblast proliferation

C2C12 cells were plated out in 96-well plates or 6-well plates at a density of 500 cells per cm^2. After 24 h cells were cultured in medium with 1000 nmol/l 25(OH)D_3 (Sigma–Aldrich), 100 nmol/l 1,25$(OH)_2D_3$ (Sigma–Aldrich) or without supplements (i.e., control). Medium was replaced every day by growth medium containing 25(OH)D_3, 1,25$(OH)_2D_3$, or control. At day 1 and 4, the proliferation of C2C12 myoblasts in the 96-well plate was measured using XTT Cell Proliferation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were incubated with XTT solution at 37°C, whereby the viable cells formed an orange formazan dye by cleaving the yellow tetrazolium salt XTT. After 2 h the orange formazan solution was quantified by a photospectrometer (Berthold Technologies, Bad Wildbad, Germany) at 450 nm. Cells from the 6-well plate were lysed and stored at −80°C until total RNA isolation.

C2C12 myoblast differentiation

C2C12 cells were plated out in 6-well plates and were grown until 90% confluence. To induce myotube formation, growth medium was changed to differentiation medium consisting of DMEM supplemented with 2% horse serum (Gibco), 10 μg/ml penicillin (Sigma–Aldrich), 10 μg/ml streptomycin (Sigma–Aldrich) and 50 μg/ml fungizone (Gibco). At day 1 and 3 of differentiation, cells were lysed and stored at −80°C until total RNA isolation or western blotting. Myotube thickness was measured at day 3 of the differentiation by obtaining images using a Leica inverted microscope type DM-IL. Subsequently, myotube thickness was determined from four images per well using ImageJ (v.1.41o, National Institute of Health, USA; http://rsbweb.nih.gov/ij/). Myotubes (>90 per experiment) were measured at three locations along their lengths (25%, 50%, and 75% of the length).

RNA isolation and RT-qPCR

Total RNA of C2C12 cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For removing residual DNA amounts an additional on-column DNase treatment was accomplished. Total RNA concentration was measured by the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

RNA was reverse transcribed from 500 ng total RNA in a 20 μl reaction mixture using the High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed using the following thermal cycler conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The PCR reaction of total 20 µl contained 5 µl cDNA, 200 nmol/l reverse and forward primer (Table 1) and SYBR Green Master Mix (Applied Biosystems). cDNA was diluted 1:10. qPCR was performed in duplicate on a StepOne real-time PCR System (Applied Biosystems): 20 sec at 95°C, 40 cycles consisting of 3 sec at 95°C, and 30 sec at 60°C. Several housekeeping genes were tested (18S, hypoxanthine phosphoribosyltransferase and glyceraldehyde 3-phosphate dehydrogenase) of which assessment of 18S rRNA expression was shown to be the more reproducible. Therefore, 18S rRNA was used as reference and the relative gene expression was calculated by the $2^{-\Delta Ct}$ method.

Table 1. Primer sequence

Target Gene	Primer sequence (5′- 3′)
CYP27B1	Forward: CATCATGGGCAGAGCACCGT
	Reverse: TCACCATCCGCCGTTAGCAA
Vitamin D receptor (VDR)	Forward: TCCTGCTCGATGCCCACCACA
	Reverse: TGCACGAATTGGAGGCCGGAA
CYP24	Forward: AACAGCACGACACACTGGCAGA
	Reverse: CTCGGCGAGCCCAGATGCAG
MyoD	Forward: CATCCAGCCCGCGCTCCAAC
	Reverse: GGGCCGCTGTAATCCATCATGCC
Myogenin	Forward: CCAGCCCATGGTGCCCAGTGA
	Reverse: CCAGTGCATTGCCCCACTCCG
PGC1α	Forward: ACACAACCGCAGTCGCAACA
	Reverse: GGGAACCCTTGGGGTCATTTGG
Ki67	Forward: GGTGGGCACCTAAGACCTGAA
	Reverse: TCCTAGGACTAGGAGCTGGAG
MHC-I (MYH7)	Forward: AGATCCGAAAGCAACTGGAG
	Reverse: CTGCCTTGATCTGGTTGAAC
MHC-IIA (MYH2)	Forward: GCAGAGACCGAGAAGGAG
	Reverse: CTTTCAAGAGGGACACCATC
MHC-IIX (MYH1)	Forward: GCGACAGACACCTCCTTCAAG
	Reverse: TCCAGCCAGCCAGCGATG
MHC-IIB (MYH4)	Forward: CAACTGAGTGAAGTGAAGACC
	Reverse: AGCTGAGAAACCATAGCGTC
MHC embryonic (MYH8)	Forward: ACTGAGGAAGACCGCAAGAA
	Reverse: CAGGTTGGCATTGGATTGTTC
18S rRNA	Forward: GTAACCCGTTGAACCCCATT
	Reverse: CCATCCAATCGGTAGTAGCG

Western blot

C2C12 cells for western blot were scraped in cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Samples (one per experiment) were denaturated in SDS–PAGE sample buffer for 5 min at 90°C, loaded onto a SDS PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). After transfer, the membrane was blocked overnight at 4°C with 2% ECL Advance Blocking Agent (GE Healthcare) in TBS with 0.01% Tween 20 (Sigma–Aldrich). Subsequently, the membrane was washed and incubated for 1 h at room temperature with primary antibody against p-Akt (Ser473; 1:4000), total Akt (1:4000), p-S6 (Ser235/236; 1:1000), total S6 (1:1000), and β-tubulin (1:2000) (all Cell Signaling Technology). After washing, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody (1:4000; DakoCytomation, Glostrup, Denmark) and the membrane was analyzed with the enhanced chemiluminescence method (ECL Advance; GE Healthcare). Western blots were quantified using ImageJ. Total Akt, p-Akt, total S6, and p-S6 were normalized to β-tubulin.

Measurement of medium concentrations of $1,25(OH)_2D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$

C2C12 myoblasts were seeded into 6-well plates and were grown until 90% confluence. Cells were induced to form myotubes by changing growth medium to differentiation medium. After 3 days of differentiation, cells were cultured in medium supplemented with 0, 400, 1000, or 2000 nmol/l $25(OH)D_3$ for 24 h. Medium was collected and stored at –20°C until measurement of vitamin D_3 metabolites. As positive control, primary human osteoblasts were cultured in medium supplemented with 0, 400, and 1000 nmol/l $25(OH)D_3$ in 6-well plates with a cell density of 500.000 cells per well for 24 h, as described previously [57]. Medium was collected and stored at –20°C until measurement of vitamin D_3 metabolites.

The metabolite $1,25(OH)_2D_3$ was measured in non-conditioned and conditioned medium using a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Cross reactivity with $25(OH)D_3$ and $24R,25(OH)_2D_3$ was 0.01% and <0.01% respectively. The intra-assay variation was 8% at a level of 25 pmol/l and 9% at a level of 70 pmol/l. The inter-assay variation was 11% at a concentration of 25 and 70 pmol/l.

The metabolites $25(OH)D_3$ and $24R,25(OH)_2D_3$ were analyzed in non-conditioned and conditioned medium using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, samples were incubated with deuterated internal vitamin D standards (d6-$25(OH)D_3$ and d6-$24R,25(OH)_2D_3$) and protein-precipitated using acetonitrile. Supernatant was, after PTAD derivatization, purified using a Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands), followed by detection with a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). Intra-assay variation of 25(OH)D_3 was 9.6%, 6.0%, and 8.5% at a level of 58, 191, and 516 nmol/l, respectively. Intra-assay variation of 24R,25$(OH)_2D_3$ was 5.4% and 9.1% at a level of 46 and 150 nmol/l, respectively.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using SPSS version 20.0 software (SPSS Inc., Chicago, IL). Differences between groups were assessed using a one-way ANOVA followed by Bonferroni's post hoc test to examine the effects of treatment on myotube diameter and myotube number. A two-way ANOVA followed by Bonferroni's post hoc test was used to examine the effects of time and vitamin D_3 treatment on absorbance values, mRNA expression levels and protein expression levels. A three-way ANOVA was used to examine whether time and vitamin D_3 treatment affect expression levels of myosin heavy chain isoforms. A p-value<0.05 was considered to be significant (*p<0.05, ** p<0.01, *** p<0.001).

RESULTS

Both 25(OH)D_3 and 1,25$(OH)_2D_3$ attenuated C2C12 myoblast proliferation

Figure 1 shows effects of 1 and 4 days of supplementation of 25(OH)D_3 and 1,25$(OH)_2D_3$ on estimates of viable cell numbers determined by the absorbance of the colored formazan product which is directly proportional to the number of viable cells. Two-way ANOVA revealed a significant effect of time as well as an interaction between time and vitamin D_3 treatment (p<0.001). As expected, for all treatment groups the number of viable cells was significantly increased at day 4 compared to day 1 (p<0.001; Fig. 1D). At day 4, both 1000 nmol/l 25(OH)D_3 and 100 nmol/l 1,25$(OH)_2D_3$ significantly reduced the number of viable cells compared to control culture (p<0.001). In the presence of 1000 nmol/l 25(OH)D_3 and 100 nmol/l 1,25$(OH)_2D_3$ the proportion of viable cells was shown to be 30.1% and 27.6% lower than in the control condition, respectively.

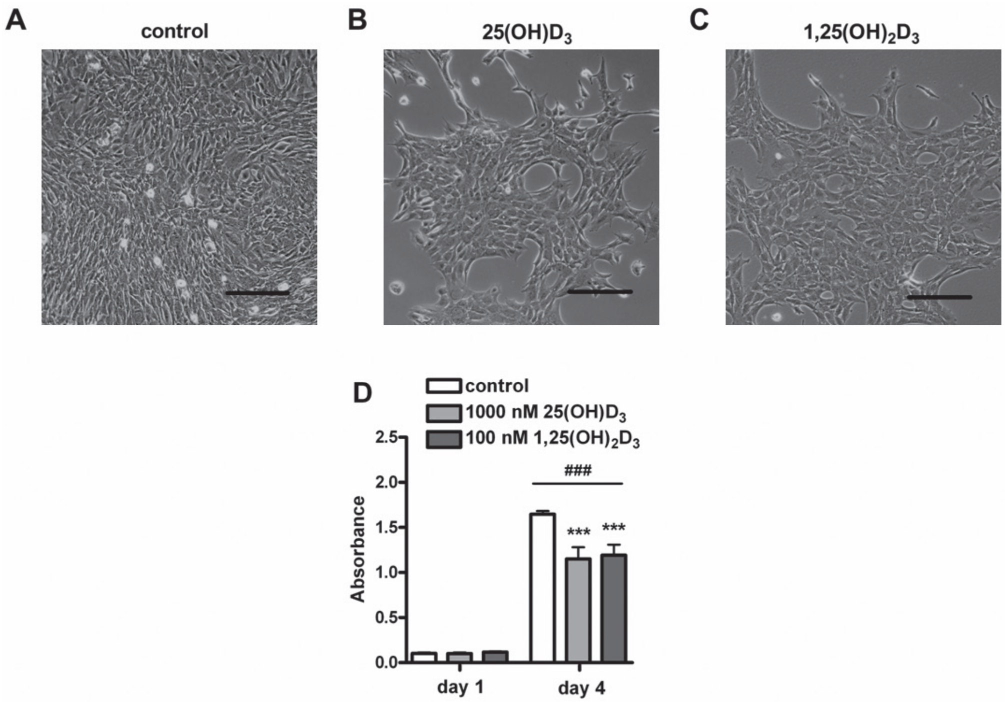

Figure 1. Both 25(OH)D_3 and 1,25$(OH)_2D_3$ attenuated C2C12 myoblast proliferation. Micrographs of C2C12 myoblasts cultured for 4 days in growth medium **(A)**, in growth medium supplemented with 1000 nmol/l 25(OH)D_3 **(B)** or 100 nmol/l 1,25$(OH)_2D_3$ **(C)**. Myoblast proliferation was quantified at day 1 and 4 **(D)**. Scale bar indicates 100 µm. Data were analyzed using a two-way ANOVA followed by Bonferroni's post-hoc comparisons test. Values are mean ± SEM (n=20). ***$p<0.001$; $^{###}p<0.001$ ($^{\#}$ between time period, * between vitamin D_3 concentrations)

Both 25(OH)D_3 and 1,25$(OH)_2D_3$ increased CYP24 and VDR mRNA levels and reduced myogenin mRNA levels in proliferating myoblasts

Since myoblast proliferation was reduced by vitamin D_3 metabolites, we next investigated whether myoblasts expressed mRNA of proteins involved in vitamin D_3 metabolism and whether expression levels were modulated by vitamin D_3 metabolites. Myoblasts did express CYP27B1, CYP24, and VDR. At day 4, CYP27B1 mRNA levels were lower compared to those at day 1 ($p<0.05$; Fig. 2A), but significant effects of 25(OH)D_3 or 1,25$(OH)_2D_3$ were not observed. CYP24 mRNA levels were higher at day 4 compared to day 1 ($p<0.01$; Fig. 2B). At day 1 and day 4, CYP24 and VDR mRNA levels were both significantly increased by 25(OH)D_3 ($p<0.01$) as well as by 1,25$(OH)_2D_3$ ($p<0.01$ and $p<0.05$, respectively; Fig. 2B and C). To investigate mechanisms underlying the anti-proliferative effects of both vitamin D_3 metabolites, we determined mRNA levels of ki67 and MyoD. Ki67 mRNA was significantly reduced at day 4 compared to day 1 ($p<0.001$; Fig. 2D), but 25(OH)D_3 and 1,25$(OH)_2D_3$ supplementation did not change these mRNA levels. MyoD mRNA levels were also not affected by 25(OH)D_3 or 1,25$(OH)_2D_3$ supplementation (Fig. 2E). For myogenin, two-way ANOVA showed a significant interaction between the effects of time and vitamin D_3 treatment ($p<0.05$; Fig. 2F). Post hoc analysis revealed that 25(OH)D_3 and 1,25(OH)$_2D_3$ reduced myogenin mRNA levels at day 4 of proliferation ($p<0.01$). In addition, myogenin mRNA levels in control cultures were significantly increased at day 4 compared to day 1 ($p<0.001$), but mRNA levels at day 4 were still 44 times lower than myogenin mRNA levels in myotubes (Fig. 4E).

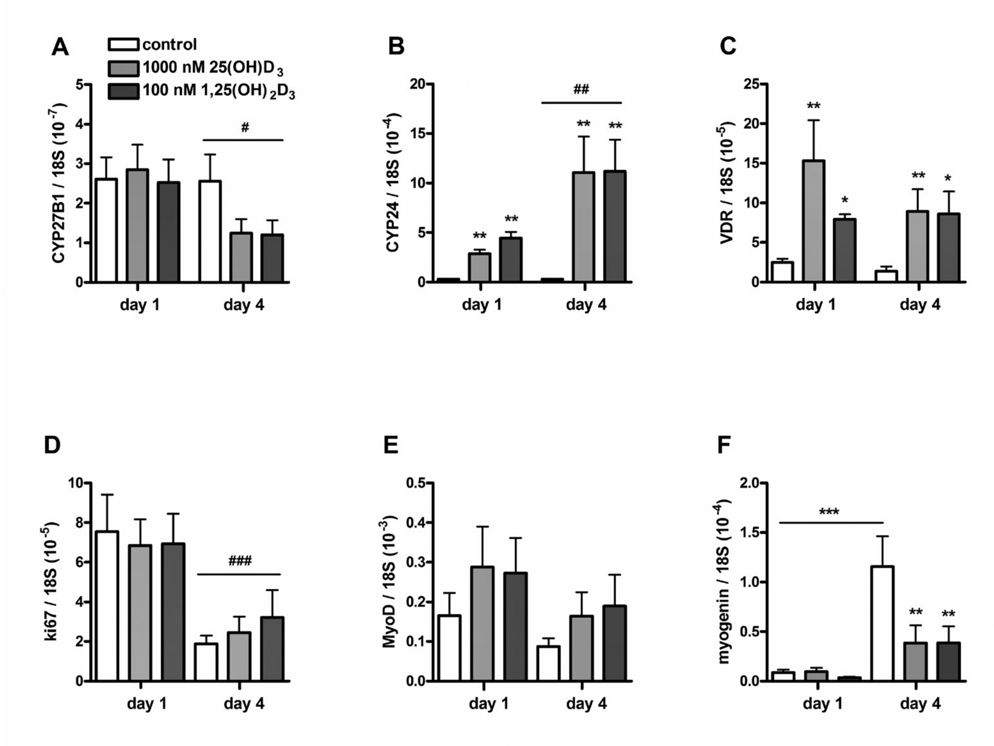

Figure 2. Both 25(OH)D_3 and 1,25(OH)$_2D_3$ increased CYP24 and VDR mRNA levels and reduced myogenin mRNA levels in myoblasts. Myoblasts were cultured for 4 days in growth medium supplemented with 1000 nmol/l 25(OH)D_3, 100 nmol/l 1,25(OH)$_2D_3$ or without any supplements. After 1 day and 4 days, mRNA levels of CYP27B1 **(A)**, CYP24 **(B)**, VDR **(C)**, ki67 **(D)**, MyoD **(E)** and myogenin **(F)** were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post-hoc comparisons test. Values are mean ± SEM (n=8). *$p<0.05$, **$p<0.01$, ***$p<0.001$; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ ($^{\#}$ between time period, * between vitamin D_3 concentrations)

Effects of 25(OH)D_3 and 1,25(OH)$_2D_3$ on myotube diameter

Confluent (90%) cultures of myoblasts differentiated into myotubes during 3 days of culture in differentiation medium. To examine whether 25(OH)D_3 and 1,25(OH)$_2D_3$ stimulated myotube hypertrophy, we measured myotube diameter at day 3 of the differentiation. Figure 3 shows micrographs of control myotubes, and 25(OH)D_3 or 1,25$(OH)_2D_3$ treated cells taken at day 3 of treatment (Fig. 3A–C). The diameter of myotubes exposed to 25(OH)D_3 was slightly increased compared to control myotubes (19%; $p<0.05$; Fig. 3D), but an effect of 1,25$(OH)_2D_3$ on myotube diameter was not observed. The number of myotubes per mm^2 was not altered by both vitamin D_3 metabolites (Fig. 3E).

To investigate whether the lack of hypertrophy was associated with the high dose of the vitamin D_3 metabolites, we tested whether low concentrations of vitamin D_3 metabolites would induce myotube hypertrophy. For this purpose myoblasts were differentiated into myotubes in the presence of 100 nmol/l 25(OH)D_3 and 1 nmol/l 1,25$(OH)_2D_3$. However, these low concentrations of 25(OH)D_3 and 1,25$(OH)_2D_3$ did not affect myotube diameter or the number of myotubes per mm^2 (sFig. 1A and B, supplementary material).

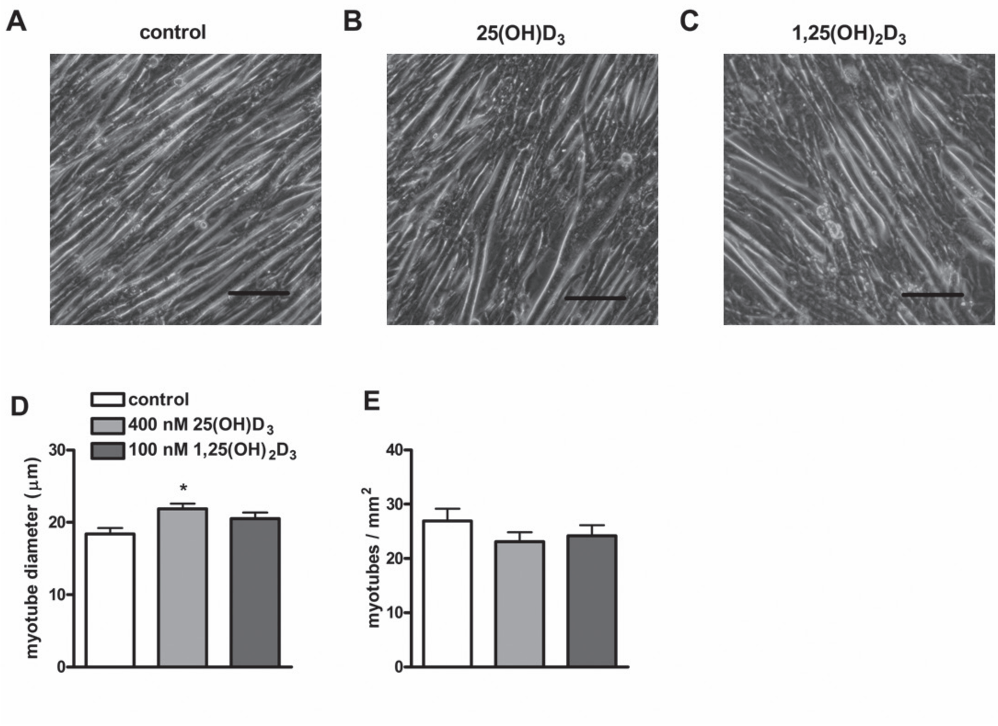

Figure 3. Effects of 25(OH)D_3 and 1,25$(OH)_2D_3$ on myotube diameter. Micrographs of C2C12 myoblasts cultured for 3 days in differentiation medium **(A)**, in differentiation medium supplemented with 400 nmol/l 25(OH)D_3 **(B)** or 100 nmol/l 1,25$(OH)_2D_3$ **(C)**. After 3 days of culture, myotube diameter (μm) **(D)** and myotubes/mm^2 **(E)** were determined. Scale bar indicates 100 μm. Data were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc test. Values are mean ± SEM (n=8). *$p<0.05$

Both 25(OH)D_3 and 1,25(OH)$_2D_3$ increased CYP24 and VDR mRNA levels in differentiating myotubes

Figure 4 shows mRNA levels of CYP27B1, CYP24, VDR, MyoD, myogenin, and PGC1α during myotube formation. CYP27B1 mRNA levels after 3 days of culture in differentiation medium were increased compared to those after 1 day ($p<0.001$; Fig. 4A). CYP24 mRNA levels were substantially increased by both 25(OH)D_3 and 1,25(OH)$_2D_3$ ($p<0.001$; Fig. 4B). For mRNA levels of VDR, two-way ANOVA showed significant interaction effects between time and vitamin D_3 treatment ($p<0.05$; Fig. 4C). Post hoc analyses showed that after 3 days of differentiation both 25(OH)D_3 and 1,25(OH)$_2D_3$ increased VDR mRNA levels ($p<0.05$ and $p<0.001$, respectively). In addition, within 1,25(OH)$_2D_3$ treated myotubes, VDR mRNA levels at day 3 were increased compared to those measured at day 1 ($p<0.001$). MyoD mRNA levels were not affected by time or vitamin D_3 treatment (Fig. 4D). After 3 days of culture in differentiation medium, myogenin and PGC1α mRNA levels were significantly higher than those after 1 day of culture in differentiation medium ($p<0.001$ and $p<0.01$, respectively; Fig.4E and F), but effects of vitamin D_3 treatment could not be shown.

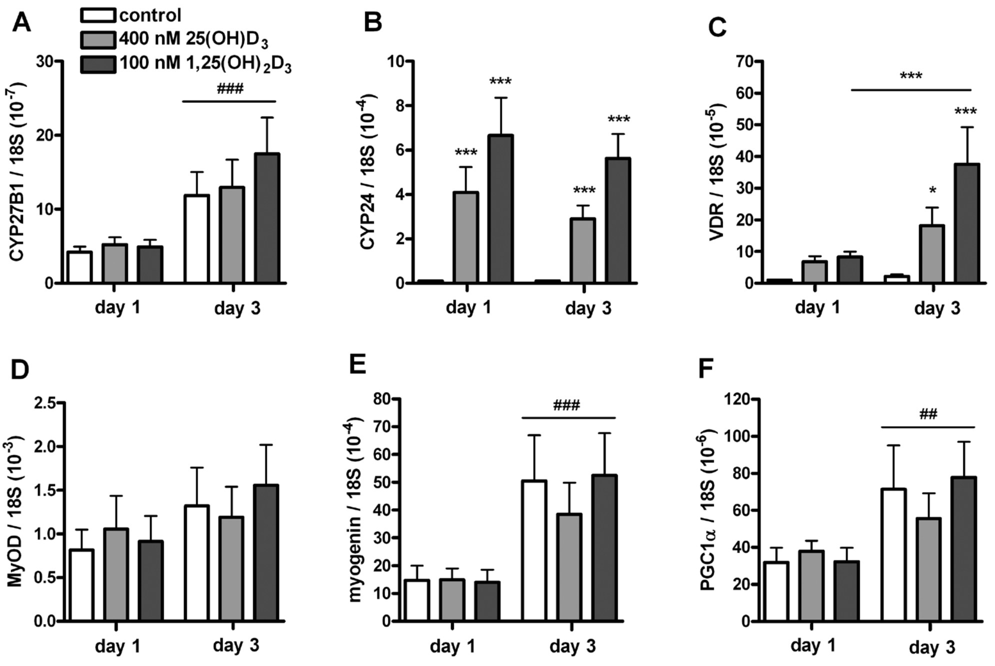

Figure 4. Both 25(OH)D_3 and 1,25(OH)$_2D_3$ increased CYP24 and VDR mRNA levels in differentiating C2C12 cells. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/l 25(OH)D_3, 100 nmol/l 1,25(OH)$_2D_3$ or without any supplements. After 1 day and 3 days of culture, mRNA levels of CYP27B1 **(A)**, CYP24 **(B)**, VDR **(C)**, MyoD **(D)**, myogenin **(E)** and PGC1α **(F)** were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post-hoc comparisons test. Values are mean ± SEM (n=6). *$p<0.05$, ***$p<0.001$; ##$p<0.01$, ###$p<0.001$ (# between time period, * between vitamin D_3 concentrations)

We also verified whether low concentrations of 25(OH)D_3 (100 nmol/l) and 1,25$(OH)_2D_3$ (1 nmol/l) were able to affect mRNA levels of CYP27B1, CYP24, VDR, MyoD, and myogenin during myotube formation (sFig. 2A–E, supplementary material). Low concentrations of 25(OH)D_3 and 1,25$(OH)_2D_3$ did not affect CYP27B1 mRNA (sFig. 2A). CYP24 and VDR mRNA levels were markedly increased by 1 nmol/l 1,25$(OH)_2D_3$ ($p<0.001$; sFig. 2B and C), but 100 nmol/l 25(OH)D_3 did not induce CYP24 or VDR mRNA. MyoD and myogenin mRNA levels were not affected by low concentrations of 25(OH)D_3 and 1,25$(OH)_2D_3$ (sFig. 2D and E).

Effects of 25(OH)D_3 and 1,25$(OH)_2D_3$ on mRNA levels of myosin heavy chain (MHC)

Using a three-way ANOVA, we tested whether vitamin D_3 metabolites altered myotube phenotype by changing MHC isoform expression. After 3 days of culture in differentiation medium, mRNA levels of MHC-I, MHC-IIA, MHC-IIX, MHC-IIB, and MHC embryonic were increased compared to those at day 1 ($p<0.001$; Fig. 5A–E). Since MHC expression levels are a hallmark of differentiation, effects of vitamin D_3 metabolites may have been obscured due to variations in MHC expression levels between experiments as the degree of differentiation may differ from experiment to experiment. Therefore, data were also normalized (treatment/control ratio). Three-way ANOVA on normalized data showed a significant interaction between time and vitamin D_3 treatment ($p<0.001$). A main effect of vitamin D_3 treatment on MHC mRNA levels was significant at day 3, but not at day 1. Post hoc analysis revealed that for all conditions MHC mRNA levels were higher at day 3 than those at day 1 ($p<0.001$) and revealed also that at day 3, 1,25$(OH)_2D_3$ significantly increased mRNA levels of MHC compared to control ($p<0.01$).

We also verified whether low concentrations of 25(OH)D_3 (100 nmol/l) and 1,25$(OH)_2D_3$ (1 nmol/l) were able to affect mRNA levels of different types of MHC during myotube formation (sFig. 3A–E, supplementary material). We found that MHC mRNA levels were higher at day 3 than those at day 1 ($p<0.05$), but low concentrations of 25(OH)D_3 and 1,25$(OH)_2D_3$ did not affect mRNA levels of any of the MHC isoforms.

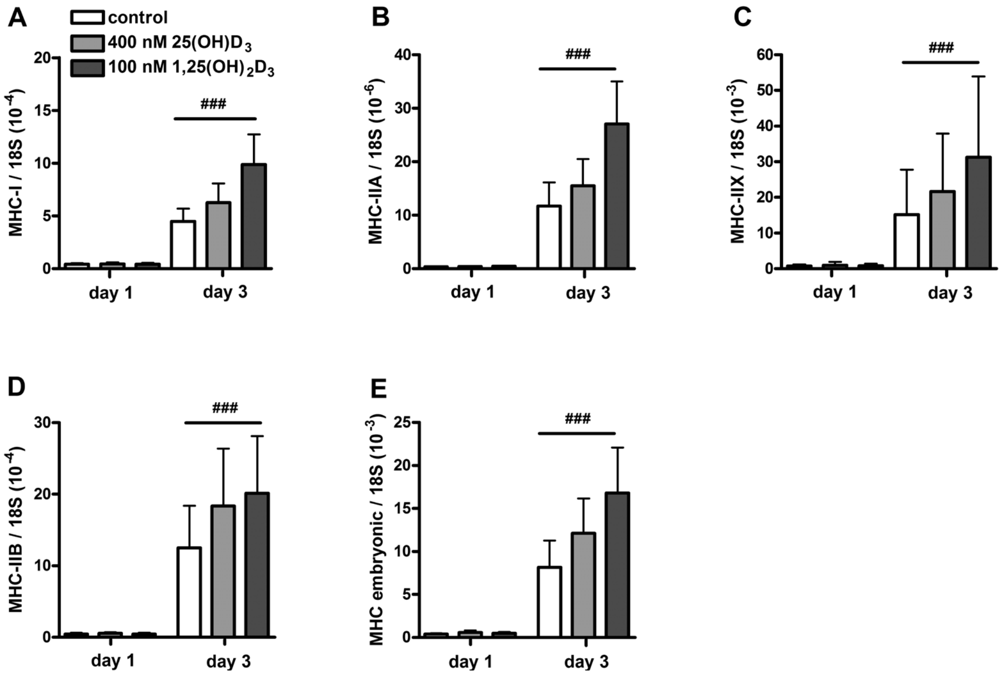

Figure 5. Effects of 25(OH)D_3 and 1,25(OH)$_2D_3$ on mRNA levels of myosin heavy chain. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/l 25(OH)D_3, 100 nmol/l 1,25(OH)$_2D_3$ or without any supplements. After 1 day and 3 days of culture, mRNA levels of MHC-I **(A)**, MHC-IIA **(B)**, MHC-IIX **(C)**, MHC-IIB **(D)** and MHC embryonic **(E)** were determined. Data were analyzed using a three-way ANOVA. Values are mean ± SEM (n=6). $^{\#\#\#}p<0.001$ ($^{\#}$ between time period)

Both 25(OH)D_3 and 1,25(OH)$_2D_3$ did not affect levels of p-Akt, total Akt, p-S6, and total S6 during myotube formation

To explain the positive effect of 25(OH)D_3 on myotube size, we examined whether 25(OH)D_3 and 1,25(OH)$_2D_3$ activate components of the Akt/mTOR signaling pathway, including Akt and S6. Levels of p-Akt and p-S6 were not affected by time and treatment with vitamin D_3 metabolites (Fig. 6A and B). Total Akt protein levels after 3 days of culture were increased compared to those at day 1 ($p<0.01$; Fig. 6C), but the two vitamin D_3 metabolites did not change this. Total S6 levels were not affected by time and vitamin D_3 treatment (Fig. 6D). Ratio of p-Akt/total Akt was lower at day 3 compared to day 1 ($p<0.01$; Fig. 6E), whereas a higher ratio of p-S6/total S6 was found at day 3 compared to day 1 ($p<0.05$; Fig. 6F). These results indicate that both vitamin D_3 metabolites did not enhance Akt/mTOR signaling.

Myotubes did not synthesize detectable 1,25(OH)$_2D_3$ levels, but synthesized 24R,25(OH)$_2D_3$ after exposure to 25(OH)D_3

To test whether the effects of 25(OH)D_3 occur via conversion to 1,25(OH)$_2D_3$, we investigated whether C2C12 cells were able to synthesize 1,25(OH)$_2D_3$ from 25(OH)D_3.

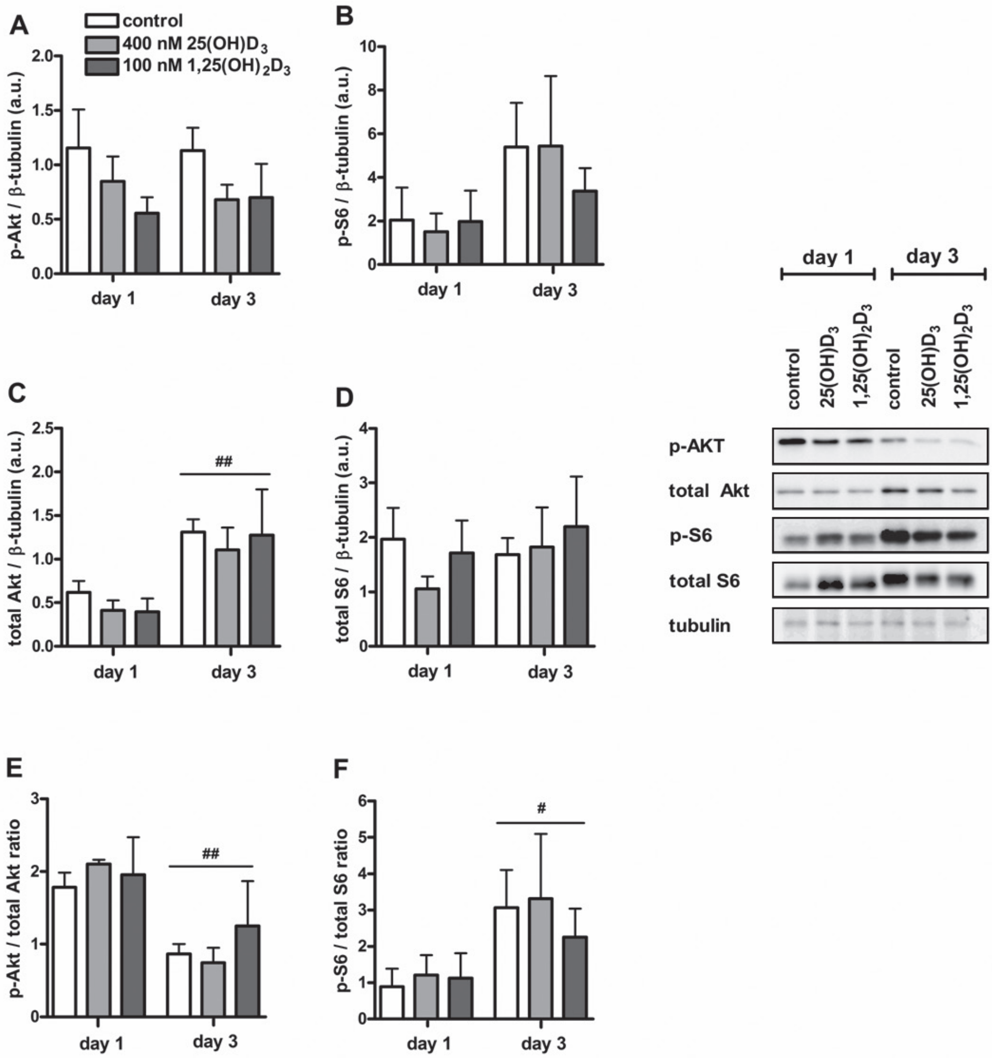

Figure 6. Both 25(OH)D_3 and 1,25$(OH)_2D_3$ did not affect levels of p-Akt, total Akt, p-S6 and total S6 in differentiating C2C12 cells. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/l 25(OH)D_3, 100 nmol/l 1,25$(OH)_2D_3$ or without any supplements. After 1 day and 3 days of culture, levels of p-Akt **(A)**, p-S6 **(B),** total Akt **(C)** and total S6 **(D)** and the ratio of p-Akt/total Akt **(E)** and p-S6/total S6 **(F)** were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post-hoc comparisons test. Values are mean ± SEM (n=4). $^{\#}p<0.05$, $^{\#\#}p<0.01$ ($^{\#}$ between time period, * between vitamin D_3 concentrations)

Because CYP27B1 mRNA levels in cells at day 3 of the differentiation (myotubes) were higher than those at day 1 (myoblasts), we chose to examine the conversion in myotubes. Myotubes were exposed to 0, 400, 1000, or 2000 nmol/l 25(OH)D_3 and after 24 h 25(OH)D_3 concentrations were strongly reduced to, respectively, 42%, 34%, and 36% of non-conditioned values (data not shown). However, after 24 h of culture the metabolite 1,25$(OH)_2D_3$ could not be detected in medium (Table 2A). As positive control, osteoblasts were cultured in medium supplemented with 0, 400, or 1000 nmol/l 25$(OH)D_3$. After 24 h, mean concentrations of, respectively, <10.0, 110.3, and 183.0 pmol/l 1,25$(OH)_2D_3$ were measured in medium, whereas mean 1,25$(OH)_2D_3$ concentrations in non-conditioned medium of, respectively, <10.0, 43.3, and 64.7 pmol/l were measured. These results indicate that primary human osteoblasts do convert 25$(OH)D_3$ to 1,25$(OH)_2D_3$, whereas C2C12 myotubes do not.

Because CYP24 mRNA levels were strongly induced after 25$(OH)D_3$ treatment, we examined whether myotubes were able to synthesize 24R,25$(OH)_2D_3$ from 25$(OH)D_3$. Myotubes were exposed to 0, 400, 1000, or 2000 nmol/l 25$(OH)D_3$ and after 24 h of culture we, respectively, measured mean concentrations of <3, 6.8, 7.4, and 14.5 nmol/l 24R,25$(OH)_2D_3$ in medium (Table 2B). In non-conditioned medium, 24R,25$(OH)_2D_3$ concentrations were below their detection limit (<3 nmol/l). As positive control, osteoblasts were cultured in medium supplemented with 0, 400, or 1000 nmol/l 25(OH) D_3. After 24 h, mean concentrations of, respectively, <3, 70.2, and 105.4 nmol/l 24R,25$(OH)_2D_3$ were measured in medium, whereas 24R,25$(OH)_2D_3$ concentrations in non-conditioned medium were below their detection limit (<3 nmol/l). These results indicate that myotubes are able to convert 25$(OH)D_3$ to 24R,25$(OH)_2D_3$.

Table 2. Myotubes did not synthesize detectable 1,25$(OH)_2D_3$ levels after exposure to 25$(OH)D_3$. Myotubes and osteoblasts (positive control) were cultured in medium supplemented with increasing concentrations of 25$(OH)D_3$. After 24 hours, 1,25$(OH)_2D_3$ (**A**) and 24R,25$(OH)_2D_3$ (**B**) concentrations in non-conditioned (PRE) and conditioned (POST) culture medium were measured. Data are presented as mean ± SEM. Regarding the osteoblast culture, concentrations of 1,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ in non-conditioned and conditioned medium have been published previously [57].

A	**MYOTUBES**		**OSTEOBLASTS positive control**	
	1,25$(OH)_2D_3$ (pM) PRE	**1,25$(OH)_2D_3$ (pM) POST**	**1,25$(OH)_2D_3$ (pM) PRE**	**1,25$(OH)_2D_3$ (pM) POST**
0 nM 25$(OH)D_3$	13.0	12.0 ± 1.0	<10.0	<10.0
400 nM 25$(OH)D_3$	43.0	30.5 ± 0.5	43.3 ± 2.2	110.3 ± 13.5
1000 nM 25$(OH)D_3$	76.0	52.5 ± 0.5	64.7 ± 4.8	183.0 ± 26.6
2000 nM 25$(OH)D_3$	125.0	105.0 ± 3.0	-	-

B	**MYOTUBES**		**OSTEOBLASTS positive control**	
	24R,25$(OH)_2D_3$ (nM) PRE	**24R,25$(OH)_2D_3$ (nM) POST**	**24R,25$(OH)_2D_3$ (nM) PRE**	**24R,25$(OH)_2D_3$ (nM) POST**
0 nM 25$(OH)D_3$	<3.0	<3.0	<3.0	<3.0
400 nM 25$(OH)D_3$	<3.0	6.8 ± 0.1	<3.0	70.2 ± 4.4
1000 nM 25$(OH)D_3$	<3.0	7.4 ± 1.3	<3.0	105.4 ± 8.2
2000 nM 25$(OH)D_3$	<3.0	14.5 ± 2.1	-	-

DISCUSSION

The aim of this study was twofold: (i) to investigate the effects of $1,25(OH)_2D_3$ and $25(OH)D_3$ on proliferation and differentiation of myoblasts and myotube size; and (ii) to investigate $25(OH)D_3$ metabolism within C2C12 muscle cells. With respect to our first aim, we demonstrated in myoblasts that both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased VDR mRNA levels, reduced proliferation and decreased myogenin mRNA levels. During differentiation, both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased VDR mRNA levels, but did not activate the Akt/mTOR pathway. Only $25(OH)D_3$ slightly increased myotube size. Regarding our second aim, we hypothesized that effects of $25(OH)D_3$ occur after its conversion to $1,25(OH)_2D_3$, but despite the presence of CYP27B1 mRNA in myoblasts and myotubes we could not demonstrate $1,25(OH)_2D_3$ synthesis in medium of myotubes after exposure to $25(OH)D_3$. Interestingly, in myoblasts and myotubes CYP24 mRNA levels were increased in response to $25(OH)D_3$ and accompanied by elevated $24R,25(OH)_2D_3$ levels in medium. These results suggest that skeletal muscle cells not only respond to vitamin D_3 metabolites, but are also able to reduce vitamin D signaling by the activity of CYP24.

Proliferation

During myoblast proliferation, VDR mRNA expression was higher in myoblasts treated with $1,25(OH)_2D_3$ than those without treatment. This observation is consistent with previous studies [22;25;46] and suggests not only the presence of genomic transcriptional effects via the VDR, but also an increased responsiveness to $1,25(OH)_2D_3$. Genomic effects of $1,25(OH)_2D_3$ via the VDR were confirmed by strongly increased mRNA levels of CYP24, which is a target gene of the VDR. Treatment with $1,25(OH)_2D_3$ also resulted in a reduction of myoblast number which is in line with several other studies [22;24;35;44;46]. This reduction of cell number in our study may in part be regulated by the genomic pathway of $1,25(OH)_2D_3$, since it has been shown that expression of cell cycle genes is altered by $1,25(OH)_2D_3$ [18;24]. In addition to genomic actions, non-genomic actions of $1,25(OH)_2D_3$ such as stimulation of ERK1/2 [39] and p38 MAPK [12] have been reported to modulate proliferation of myoblasts. In addition to the inhibitory effects of $1,25(OH)_2D_3$, a few studies reported a stimulatory effect of $1,25(OH)_2D_3$ [5;11]. These stimulatory effects of $1,25(OH)_2D_3$ on myoblast proliferation were only demonstrated at early time-points (4–24 h), whereas inhibitory effects of $1,25(OH)_2D_3$ were mainly found at later time-points suggesting that the effect of $1,25(OH)_2D_3$ on proliferation is time-dependent. Serum concentration in medium is also important for the effects of $1,25(OH)_2D_3$ on proliferation, since it has been reported that $1,25(OH)_2D_3$ induces inhibitory effects in cultures with lower serum concentrations (5–10%), while higher serum concentrations (15–20%) result in stimulatory effects [17]. Both factors, serum and time, do probably affect the differentiation state of the cell which may determine the response of the cell to

$1,25(OH)_2D_3$. Furthermore, we observed that myoblast number was not only lower after treatment with $1,25(OH)_2D_3$, but also after treatment with its precursor $25(OH)D_3$. This result confirms recent studies that found an anti-proliferative effect of $25(OH)D_3$ on myoblasts as well [24;46]. It shows that muscle cells have the capacity to take up $25(OH)D_3$ [1] and that $25(OH)D_3$ is directly or indirectly able to trigger mechanisms to reduce cell number or inhibit proliferation. Effects of $25(OH)D_3$ may occur via conversion to $1,25(OH)_2D_3$ since C2C12 myoblasts express CYP27B1, however as our results on myotubes show that myotubes do not convert $25(OH)D_3$ to $1,25(OH)_2D_3$ a direct effect may also be possible. Although $25(OH)D_3$ has a low affinity for the VDR [31], supra-physiological concentrations of $25(OH)D_3$ may activate the VDR leading to altered gene expression levels.

MyoD and ki67 were not significantly affected by both $25(OH)D_3$ or $1,25(OH)_2D_3$, but myogenin mRNA levels were lower after treatment with both metabolites compared to non-treated myoblasts which suggests that $25(OH)D_3$ and $1,25(OH)_2D_3$ inhibit the differentiation in growth medium. However, it is also possible that the higher mRNA levels of myogenin in control cultures were due to the almost confluent cell culture at the end of the proliferation experiment. An increased cell density will lead to more cell–cell contact which results in an earlier initiation of the differentiation [34].

Differentiation and hypertrophy

As in myoblasts, differentiated myotubes also showed increased VDR mRNA levels by treatment with $25(OH)D_3$ or $1,25(OH)D_3$, suggesting the presence of genomic transcriptional effects via the VDR. However, vitamin D_3 signaling did not result in hypertrophic effects; we observed only a minor increase in myotube diameter (19% in 3 days) by $25(OH)D_3$. Other studies demonstrated increases in myotube diameter by 80–100% after $1,25(OH)_2D_3$ or $25(OH)D_3$ treatment [22;24]. Differences in experimental set up may clarify the conflicting results. Studies which observed an effect of $1,25(OH)_2D_3$ or $25(OH)D_3$ on myotube diameter, used a prolonged cell culture model in which proliferation was immediately followed by myotube formation. Due to anti-proliferative effects of $1,25(OH)_2D_3$ or $25(OH)D_3$, a lower number of cells was present at the start of the differentiation in myotubes in the cultures that had been treated with $1,25(OH)_2D_3$ or $25(OH)D_3$ compared to cell cultures without treatment. The lower number of myoblasts may have resulted in a lower number of myotubes, and thicker myotubes due to extra space in the culture well. This hypothesis is supported by data showing an optimal seeding density of human myoblasts in a 3D engineered collagen construct to obtain maximal force production of myotubes [34]. A high myoblast density may have a negative impact on myoblast force generating capacity and is associated with slow myosin expression [34]. In our study, myotube formation was investigated after starting with the same cell number and we did not observe any effect of $1,25(OH)_2D_3$ and only a minor effect of $25(OH)D_3$ on myotube number and size suggesting that both metabolites are not potent hypertrophic agents like for instance insulin-like growth factor-1 (IGF-1) [49]. To verify whether the lack of substantial hypertrophy was due to a lack of hypertrophic signaling, we investigated the activity of the Akt/mTOR signaling pathway, a key pathway involved in skeletal myotube hypertrophy [60] in response to $1{,}25(OH)_2D_3$ and $25(OH)D_3$. Akt mediates a wide range of cellular functions including cell proliferation, differentiation, gene transcription, and the rate of mRNA translation [9;23;26]. Akt activation has been demonstrated by $1{,}25(OH)_2D_3$ during proliferation and differentiation of C2C12 myoblasts [10,11]. However, in our study during differentiation no effect of $1{,}25(OH)_2D_3$ or $25(OH)D_3$ on the phosphorylation of Akt was observed. Moreover, downstream p-S6 was also not affected by both metabolites. Therefore, these results suggest that both $1{,}25(OH)_2D_3$ and $25(OH)D_3$ did not activate the Akt/mTOR signaling pathway in our model. These observations are in line with those of an in vivo rat study in which supra-physiological $1{,}25(OH)_2D_3$ levels did not result in muscle hypertrophy, but rather in muscle atrophy [52]. This in vivo negative effect on muscle mass could be indirect, but in our cell culture model we investigated direct hypertrophic effects of $1{,}25(OH)_2D_3$ and $25(OH)D_3$ which were not present.

Expression of transcription factors that are essential for differentiation, including MyoD and myogenin, were also not affected by $25(OH)D_3$ or $1{,}25(OH)_2D_3$. However, at day 3 of differentiation MHC mRNA levels were increased by high concentrations of $1{,}25(OH)_2D_3$. Effects of $1{,}25(OH)_2D_3$ on MHC expression levels have been reported before, but those were not consistent [35;51]. In differentiating C2C12 myoblasts $1{,}25(OH)_2D_3$ decreased embryonic MHC, while in C2C12 differentiated myotubes $1{,}25(OH)_2D_3$ increased MHC-IIA mRNA expression [35]. In vivo injection of $1{,}25(OH)_2D$ in steers also showed an increased MHC-IIA expression [29]. In contrast, in differentiating C2C12 myoblasts $1{,}25(OH)_2D_3$ supplementation has also been reported to increase mRNA levels of MHC-I, MHC-IIB, and MHC embryonic, without an effect on type IIA mRNA [51]. Such differences may be due to differences in differentiation phases, medium composition or species differences [35;51]. In our study, $1{,}25(OH)_2D_3$ increased MHC mRNA levels in general, however an effect on myotube diameter was not observed. This suggests that the mRNA availability was sufficient and that the rate of mRNA translation was likely not affected yet. The mechanism by which $1{,}25(OH)_2D$ affects mRNA levels of MHC is not fully elucidated. Direct regulation of MHC mRNA levels by $1{,}25(OH)_2D$ is possible through binding to its receptor [51], but non-genomic actions of $1{,}25(OH)_2D$ such as the increase in intracellular calcium concentrations [16] may also play indirectly a role in the regulation of MHC mRNA expression. Thus, based on our results and those reported in above mentioned studies, we conclude that $1{,}25(OH)_2D$ is able to increase mRNA levels of MHC isoforms, however effects seem to be determined by multiple factors. Effects of $25(OH)D_3$ on MHC expression were not observed.

Vitamin D_3 metabolism

CYP27B1 mRNA and protein expression have recently been shown in C2C12 myoblasts and C2C12 myotubes [24;25] as well as in primary murine myotubes [25] and regenerating murine muscle fibers in vivo [46]. We confirmed the presence of CYP27B1 mRNA levels in both myoblasts and myotubes. Moreover, we also showed that myotubes have even higher levels of CYP27B1 mRNA compared to myoblasts, which suggests that myotubes were able to synthesize higher quantities of $1,25(OH)_2D_3$ than myoblasts. In addition, myotubes also have a higher uptake of $25(OH)D_3$ than myoblasts [1]. However, myotubes exposed to $25(OH)D_3$ did not synthesize detectable levels of $1,25(OH)_2D_3$. This is an unexpected finding as the presence of functional CYP27B1 has been reported in C2C12 myoblasts and primary mouse myotubes by performing luciferase reporter studies [24;25]. Furthermore, it has been shown that CYP27B1 knockdown in C2C12 myoblasts abolishes the anti-proliferative effects of $25(OH)D_3$ [22], which suggests that CYP27B1 is required for the actions of $25(OH)D_3$. The questions arises why in muscle cells in our study the presence of 1α-hydroxylase activity did not result in the synthesis of detectable $1,25(OH)_2D_3$ after $25(OH)D_3$ treatment. A possible explanation is that $1,25(OH)_2D_3$ was soon converted to $1,24R,25(OH)_3D_3$ by 24-hydroxylase, which is supported by the finding that CYP24 mRNA levels were strongly increased by $1,25(OH)_2D_3$. This explanation is supported by the observation that after 24 h $25(OH)D_3$ levels were strongly reduced to 34–42% of the non-conditioned concentrations, suggesting the presence of a very high vitamin D_3 metabolism in muscle cells. In osteoblasts, $25(OH)D_3$ levels are also reduced to 16–33% of non-conditioned concentrations [57], but medium of these osteoblasts did show detectable levels of $1,25(OH)_2D_3$. Therefore, it is also possible that in C2C12 muscle cells 1α-hydroxylase activity was inhibited causing extremely low or absent $1,25(OH)_2D_3$ levels. In primary human osteoblast cultures, medium was supplemented with bovine serum albumin (BSA), but medium used in C2C12 cell cultures was supplemented with horse serum which may contain inhibiting factors such as transforming growth factor-β (TGF-β) [55] or growth factor independent-1 (GFI-1) [19]. Another explanation for the absent $1,25(OH)_2D_3$ levels in C2C12 cells may be a loss of 1α-hydroxylase activity due to post-transcriptional abnormalities or deficient cofactors such as ferredoxin reductase or ferredoxin [27].

In addition to CYP27B1 expression, myoblasts and myotubes also expressed CYP24 mRNA. We show that $25(OH)D_3$ strongly increased CYP24 mRNA in myotubes and that myotubes were able to metabolize $25(OH)D_3$ to $24R,25(OH)_2D_3$. This result shows that muscle cells have a functional enzyme, that is, 24-hydroxylase, to regulate local $25(OH)D_3$ and and $1,25(OH)_2D_3$ concentrations. The 24-hydroxylase has been proposed to be responsible for the first step in degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$, but several studies demonstrate that $24R,25(OH)_2D_3$ and $1,24R,25(OH)_3D_3$ may also play a role in bone tissue [20;21;42;59;62;63]. The metabolites $24R,25(OH)_2D_3$ and $1,24R,25(OH)_3D_3$ stimulate osteoblast differentiation in vitro [57;59]. This raises the question whether the synthesized 24R,25$(OH)_2D_3$ from 25$(OH)D_3$ in our model is able to affect myoblast proliferation and differentiation. To the best of our knowledge, there is no literature available about 24R,25$(OH)_2D_3$ actions on skeletal muscle cell proliferation and differentiation. Only in cardiac and vascular smooth muscle cells actions of 24R,25$(OH)_2D_3$ have been reported, but these actions are all associated with calcium uptake by the cells and not with myogenesis. In vascular smooth muscle cells, 24R,25$(OH)_2D_3$ is able to stimulate Ca^{2+}-ATPase and to reduce membrane L-type calcium channel activity as well as the intracellular calcium concentration [43]. In cardiac myocytes, 24R,25$(OH)_2D_3$ stimulates the calcium uptake by these cells, but less efficiently than 1,25$(OH)_2D_3$ [41]. Thus, it is possible that 24R,25$(OH)_2D_3$ affects calcium uptake by skeletal muscle cells. Regarding the actions of 24R,25$(OH)_2D_3$ in bone cells [57;59], it is also possible that 24R,25$(OH)_2D_3$ plays a role in skeletal muscle cell development or regeneration. Therefore, additional research is needed to investigate whether 24R,25$(OH)_2D_3$ affects calcium uptake by skeletal muscle cells as well as skeletal muscle cell proliferation, differentiation and hypertrophy.

5

Limitations

Doses of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ used in this study were relatively high compared to normal serum concentrations and care should be taken in the translation of these results to in vivo. However, differentiation experiments were also performed with low concentrations of 1,25$(OH)_2D_3$ (1 nM) and 25$(OH)D_3$ (100 nM) (see sFig. 1–3, supplementary material). Low concentrations of 1,25$(OH)_2D_3$ increased VDR and CYP24 mRNA levels in differentiating myoblasts similarly as the higher concentrations. Incubation of differentiating myoblasts with low concentrations of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ did not lead to altered MyoD or myogenin mRNA levels nor to myotube hypertrophy. Thus, low concentrations of 1,25$(OH)_2D_3$ and 25$(OH)D_3$ also did not have marked effects in our cell culture model with respect to differentiation and myotube size. Regarding the rate of proliferation, the anti-proliferative effects of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ at lower concentrations have been reported previously in literature (1–100 nmol/l 25$(OH)D_3$; 1–100 nmol/l 1,25$(OH)_2D_3$) [24;35]. Note, however, that tissue concentrations of 1,25$(OH)_2D_3$ can be higher than serum concentrations because of the local conversion of 25$(OH)D_3$ to 1,25$(OH)_2D_3$. The use of relatively high doses of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ did allow us to compare our results with those from other studies using C2C12 myoblasts and myotubes [22;24;46].

The metabolite 1,25$(OH)_2D_3$ was not detected in medium after treatment of C2C12 myotubes with 25$(OH)D_3$ which was probably due to very fast vitamin D_3 metabolism in these cells. Synthesized 1,25$(OH)_2D_3$ may be rapidly converted to 1,24R,25$(OH)_3D_3$ and therefore further research is needed to examine whether there is detectable 1,25$(OH)_2D_3$ synthesis on earlier time points.

Conclusion

This in vitro study shows that C2C12 myoblasts not only respond to 1,25(OH)$_2$D$_3$, but also to the precursor 25(OH)D$_3$ by reducing their proliferation and increasing their VDR expression. In differentiating myoblasts and myotubes, 1,25(OH)$_2$D$_3$ as well as 25(OH)D$_3$ stimulate VDR mRNA and in myotubes 1,25(OH)$_2$D$_3$ also stimulates MHC mRNA expression. However, this occurs without notable effects on expression of myogenic regulatory factors and myotube size. Interestingly, C2C12 myoblasts and myotubes express CYP27B1 and CYP24 mRNA which are required for vitamin D$_3$ metabolism. Although CYP27B1 activity could not be shown in myotubes, after treatment with 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$ C2C12 muscle cells showed strongly increased CYP24 mRNA levels and were able to synthesize 24R,25(OH)$_2$D$_3$ from 25(OH)D$_3$. Since 24R,25(OH)$_2$D$_3$ stimulates osteoblast differentiation in vitro, this metabolite may play a role in myoblast differentiation as well. These data suggest that skeletal muscle is not only a direct target for vitamin D$_3$ metabolites, but is also able to metabolize 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$.

Acknowledgements

We thank Huib van Essen for his excellent technical advice. We also thank the technicians of the Endocrine Laboratory for performing 1,25(OH)$_2$D$_3$ measurements.

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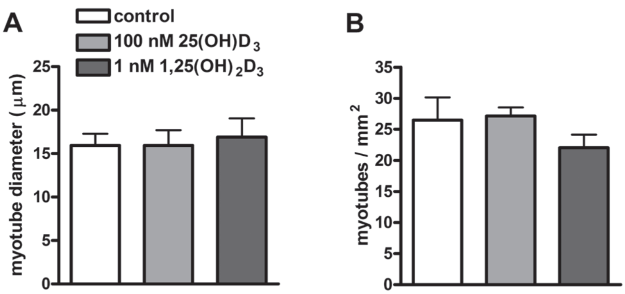
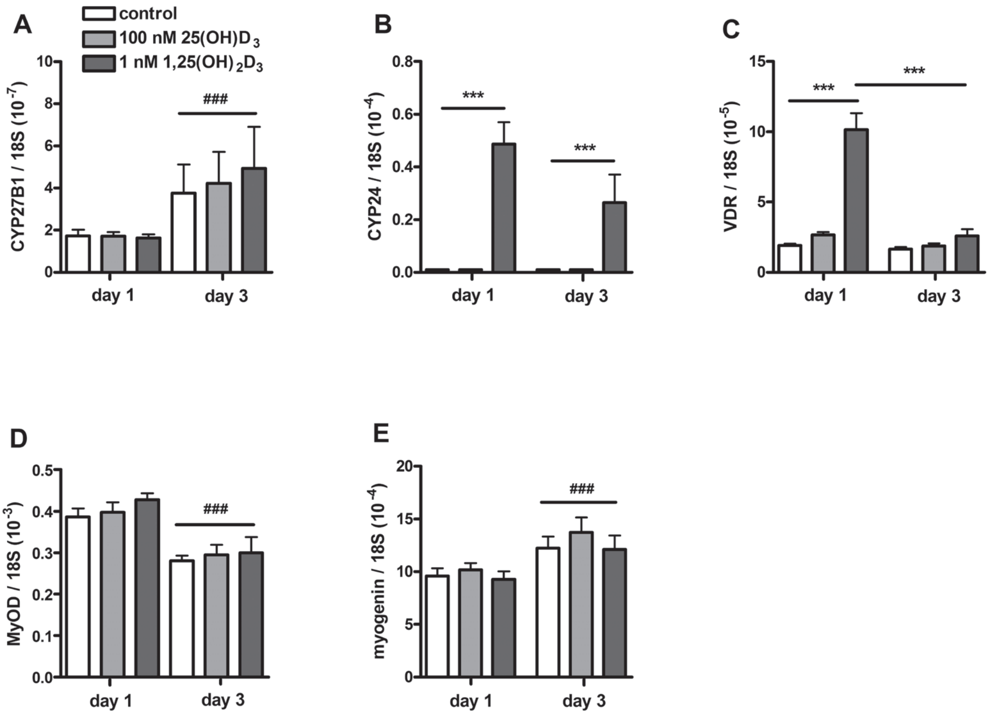
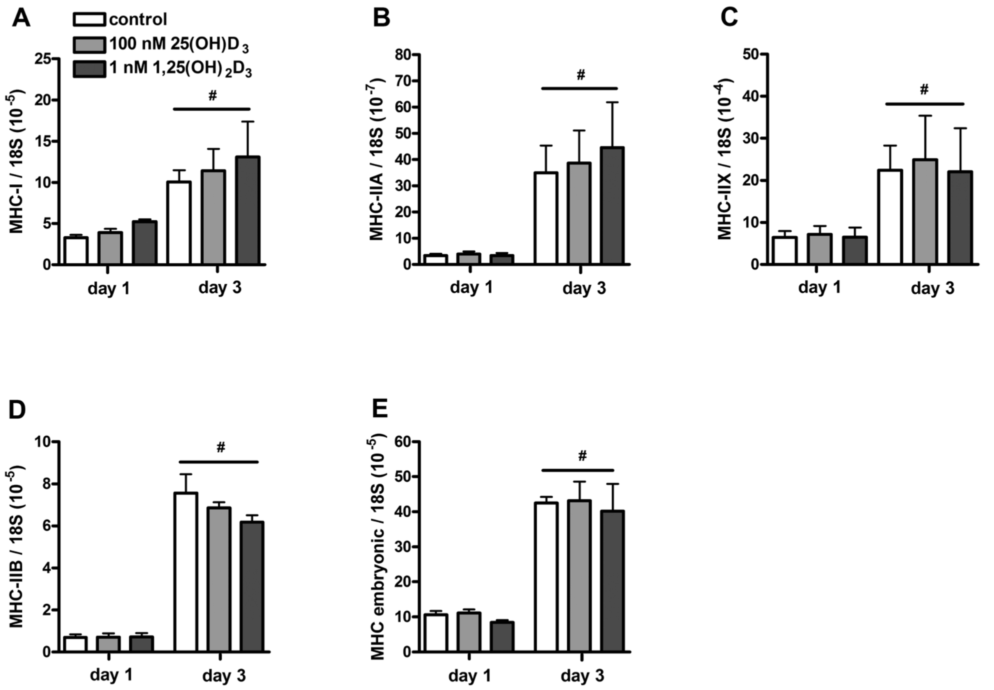
SUPPLEMENTARY MATERIAL



sFigure 1. Effects of low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ on myotube diameter. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 100 nmol/l $25(OH)D_3$, 1 nmol/l $1,25(OH)_2D_3$ or without any supplements. After 3 days of culture, myotube diameter (µm) **(A)** and myotubes/mm² **(B)** were determined. Data were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc test. Values are mean ± SEM (n=4).



sFigure 2. Low concentrations of $1,25(OH)_2D_3$ increased CYP24 and VDR mRNA levels in differentiating C2C12 cells. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 100 nmol/l $25(OH)D_3$, 1 nmol/l $1,25(OH)_2D_3$ or without any supplements. After 1 day and 3 days of culture, mRNA levels of CYP27B1 **(A)**, CYP24 **(B)**, VDR **(C)**, MyoD **(D)** and myogenin **(E)** were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post-hoc comparisons test. Values are mean ± SEM (n=4). ***$p<0.001$; ###$p<0.001$ (# between time period, * between vitamin D_3 concentrations)



sFigure 3. Effects of low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ on mRNA levels of myosin heavy chain. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 100 nmol/l $25(OH)D_3$, 1 nmol/l $1,25(OH)_2D_3$ or without any supplements. After 1 day and 3 days of culture, mRNA levels of MHC-I **(A)**, MHC-IIA **(B)**, MHC-IIX **(C)**, MHC-IIB **(D)** and MHC embryonic **(E)** were determined. Data were analyzed using a three-way ANOVA. Values are mean ± SEM (n=4). $^{\#}p<0.001$ ($^{\#}$ between time period)